NIEHS
**Burgents, Joseph**
Postdoctoral Fellow
Immunology - General
*CD103+ and CD11b+ pulmonary dendritic cells display distinct migratory properties during steady-state and following allergic sensitization*
Pulmonary dendritic cells (DC) sample inhaled antigens and initiate adaptive immune responses upon migration to lung draining lymph nodes (LN). In the lung, there are two major subsets of CD11c+ DCs, displaying high levels of CD103 (CD103 DCs) and CD11b (CD11b DCs), respectively. The migratory properties of these DC subsets remain unclear, in part because fluorescent reagents intended to label lung resident DCs can passively diffuse from the lung to draining LNs where they are taken up by LN DCs. Also, antibodies to chemokine receptors, such as CCR7, are reported to have limited specificity. To overcome these technical difficulties, we have used an alipathic fluorescent dye (PKH26) which stains pulmonary DCs, but does not diffuse to LNs, and we have generated a CCR7-gfp 'knock-in' reporter mouse strain to track display of this receptor. We report here that although both DC subsets take up Alexa fluor-tagged ovalbumin, many more CD103 DCs migrate to draining LNs than do CD11b DCs. In agreement with these differences, CCR7-gfp was expressed on more CD103 DCs and was expressed at higher levels on these cells than on CD11b DCs. This difference in CCR7-gfp expression was confirmed by analysis of CCR7-specific mRNA and by ex vivo chemotaxis of the two DC subsets in response to the CCR7 ligands CCL19 and CCL21. As expected, instillation of LPS into the lung led to the recruitment of CD11b-expressing, monocyte-derived inflammatory DCs. Surprisingly, however, these DCs did not express CCR7-gfp, and very few PKH26-labeled inflammatory DCs migrated from the lung to draining lymph nodes. Previous studies have shown that lung resident CD11b DCs can be derived either from CCR2-expressing monocytes or from CCR2- pre-DCs. Our current findings suggested the possibility that only pre-DC-derived DCs express CCR7 and are able migrate to draining lymph nodes. In agreement with this notion, CD11b DCs from CCR2-deficient mice had higher levels of CCR7 and migrated more readily to lymph nodes than did their counterparts from WT mice. Thus, CD103 DCs comprise the vast majority of migrating pulmonary DCs during both steady state and inflammatory conditions, and the relatively few CCR7-expressing CD11b DCs in the lung are likely derived from pre-DCs. These findings add to our understanding of pulmonary DC mediated immune responses and suggest that the different functions of the two major DC subsets in the lung can be explained in part by their different migratory abilities.

NIEHS
**Cavanaugh, Nisha**
Postdoctoral Fellow
Biochemistry - Proteins
*New Insights into Ribonucleotide Discrimination by DNA Polymerase Beta*
To maintain the integrity of the genome, DNA polymerases must select the correct deoxynucleotide from a pool of structurally similar substrates. This is especially challenging since ribonucleotides differ by a single hydroxyl at C2′ of the sugar and are present at much higher concentrations in the cell. Misinsertion of ribonucleotides during DNA replication or repair would render the sugar-phosphate backbone susceptible to cleavage by RNase or alkaline hydrolysis. Most DNA polymerases employ a side chain (glutamate or tyrosine) to sterically block the 2′-OH of ribonucleotides; alanine substitution of

these residues dramatically decreases ribonucleotide discrimination by the mutant polymerases. In contrast, the protein backbone of DNA polymerase beta (X–family) approaches C2´ of the sugar and the side chain of Tyr-271 forms a hydrogen bond with the primer terminus in the crystallographic structure of the closed substrate complex. To address the role tyrosine-271 plays in ribonucleotide discrimination by DNA polymerase beta, mutants were kinetically analyzed using single-nucleotide incorporation assays. Steady-state kinetic parameters revealed that alanine substitution of Tyr-271 (i.e., Y271A) had little effect on correct and incorrect insertion of deoxynucleotides, but resulted in a >10-fold loss in ribonucleotide discrimination. Although alanine substitution results in a stronger loss of discrimination with DNA polymerases from other families, the modest loss of discrimination with the Y271A mutant is consistent with the difficulty in modifying a protein backbone interaction through site-directed mutagenesis. The altered ribonucleotide discrimination was specific to Tyr-271 in DNA polymerase beta since alanine substitution of the neighboring residue, Phe-272 (i.e., F272A), had little effect on discrimination. Moreover, addition of the benzyl side chain (i.e., Y271F) only partially restored ribonucleotide discrimination, suggesting that the hydrogen bond between the Tyr-271 side chain and the primer terminus also contributes to discrimination. In summary, we propose a model where DNA polymerase beta discriminates against ribonucleotides using Tyr-271 by simultaneously blocking the 2´-OH of the sugar via the protein backbone and also altering the position of the primer terminus when forming a closed substrate complex.

NIEHS
**chang, xiaoqing**
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
*A Physiologically Based Pharmacokinetic Model of Micro and Nano Sized Fluorescent Polystyrene Spheres in Rats*
Human exposure to engineered nanoparticles has increased dramatically over the past decade. However, their potential adverse effects on human health still remain uncertain. To assess nanotoxicities, it is important to understand the pharmacokinetics of nanopartices. Physiologically-based pharmacokinetic (PBPK) modeling is a powerful tool for understanding the unique kinetic properties of nanoparticles. The lymphatic and reticuloendothelial systems (RES) have been shown to play important roles in particle uptake and sequestration. However, none of the currently available PBPK models for nanoparticles take into account these two components. In our study, we developed a PBPK model incorporating both lymphatic system and RES with a focus on toxicokinetic comparisons between nanoparticles and chemically identical micro particles. Rats were exposed to four different sizes of fluorescent polystyrene spheres (1000, 100, 40, 20 nm) via a single intravenous (IV) injection or oral pharyngeal aspiration (PA) into the airways followed by tissue distribution assessment over 90 days. A diffusion-limited, whole-body model was developed based on these data incorporating lymphatic system, macrophage phagocytosis and plasma protein binding. Using a step down procedure, we fit the data for 1000 nm particles and used this model to predict kinetics for the smaller particles. The 1000 nm model adequately predicted tissue kinetics for the three nano sized particles in liver, lung, and heart for IV exposure. The 1000 nm model also predicted tissue kinetics well in lung, liver and gastrointestinal tract for PA exposure. However, the prediction from micro to nano failed in most of the other organs, suggesting a mixture of common and uncommon kinetic mechanisms of these particles across different organs. Through a process of elimination using statistical likelihoods, we further identified modifications to the 1000 nm model providing an adequate fit to the 100 nm data. The process was repeated at each subsequent size to identify differences in kinetics between particles. A comparison of model parameters optimized for each sized particle indicated that the RES plays a major role in determining particle distribution; and that the smaller sized particle moved more freely between blood and tissues. In

summary, this work provides a general framework for elucidating the kinetics of nanoparticles and should greatly enhance our understanding of nanotoxicity.

NIEHS
**CHATTERJEE, SAURABH**
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
*Leptin signaling synergizes environmental bromodichloromethane exposure-induced post translational protein oxidations, antigen presentation and exacerbation of steatohepatitis of obesity*
Although direct exposure to high doses of disinfection byproducts like bromodichloromethane (CHBrCl2) is rare, low exposures from the environment are not uncommon especially from domestic water supply, swimming pools and ground water seepage. We tested the hypothesis that metabolic oxidative stress arising from such low exposures in obese mice synergizes with high leptin levels in these individuals and leads to exacerbation of steatohepatitis (inflammation of steatotic liver) through lipid peroxidation, post translational protein oxidations and triggering of innate immune mechanisms. Results indicated that low CHBrCl2 exposure (2mMoles/kg) caused significantly increased lipid peroxidation in hepatocytes of diet-induced obese (DIO) and leptin-knockout mice at 6h. There was also a significant increase in protein radical adducts and post-translational tyrosine nitration (from the reaction of the tyrosyl radical with nitrogen dioxide radical) in sinusoidal cells including CD68 positive Kupffer cells at 24 hrs when compared to leptin-knockout or lean control mice at 24h. The role of NADPH oxidase in free radical damage was confirmed using apocynin, a specific inhibitor for this enzyme. To study the role of the molecular basis of leptin-induced redox signaling in CHBrCl2-exposed obese mice, isolated Kupffer cells that were trans-cultured with hepatocytes from wild-type and leptin-knockout mice were probed for TNF-alpha secretion, a hallmark of macrophage activation and antigen presentation. Results showed that Kupffer cells from DIO mice exposed to CHBrCl2 had enhanced TNF-alpha release and increased MHC Class II expression, when compared to corresponding lean controls and leptin-knockout mice. Kupffer cells co-incubated either with 4-hydroxy Tempol, a superoxide dismutase mimetic, or higher concentrations of the spin trap DMPO (for freezing the radical reaction) significantly reduced macrophage activation and MHC Class II expression. Further recombinant leptin supplementation in leptin knockout mice and using SC-409, a MAPK-P38 inhibitor identified MAPK-P38 as a downstream signaling molecule for leptin action in this model. Taken together, our data for the first time suggest that CHBrCl2 bioactivation from the environment and higher circulating leptin generates an inflammatory cascade and free radical damage in obese mice. These events can be predictable causes for exacerbation of stetaohepatitis in obesity.

NIEHS
**Chowdhury, Saiful**
Research Fellow
Metabolomics/Proteomics
*Proteomic analysis of lipid rafts from ATP Binding Cassette Transporter A1-deficient macrophages reveals novel regulatory events in the innate immune response*
Lipid rafts are cholesterol-enriched membrane microdomains that dynamically organize cellular signaling events triggered by extracellular stimuli. Signaling proteins are thought to localize to rafts in part due to raft cholesterol content. The cholesterol efflux transporter ATP Binding Cassette A1 (ABCA1) acts as a primary gatekeeper for eliminating excess cholesterol from the macrophage and its rafts. It is reported that ABCA1 modulates the macrophage's signaling response to bacterial lipopolysaccharide (LPS) through remodeling of raft lipid and protein constituents. However, no proteome-scale analysis of regulation of raft composition by ABCA1 has been reported. Identification of the impact of ABCA1 on the

raft proteome of LPS-exposed macrophages carries the promise of identifying novel mechanisms of innate immunity. In order to investigate this question, rafts were isolated using detergent lysis/sucrose density gradient centrifugation from three bone marrow-derived murine macrophage preparations: 1) Abca1+/+ treated with buffer (control); 2) Abca1+/+ treated with LPS (30 min); 3) Abca1-/- treated with buffer; and 4) Abca1-/- treated with LPS. Immunoblotting for raft (flotillin-1) and non-raft (transferrin receptor) targets identified raft fractions displaying no detectable non-raft contamination. Sypro ruby staining of SDS-PAGE-resolved raft samples revealed distinct banding patterns for rafts from the two genotypes, indicating ABCA1-dependent alterations in protein expression. In-gel tryptic digestion of the three raft conditions was carried out, followed by global protein identification using LC-MS/MS and in silico database analysis (Agilent SpectrumMill software). A total 383 proteins were identified across three biological replicates with a minimum of 2 peptides and with a 0.2 % protein FDR and 2% peptide FDR. Several proteins exclusively recruited to rafts have been identified in the LPS-treated and Abca1-deleted samples, including phospholipase C alpha, C5 component of proteosome, and histocompatibility complex. The effect of LPS on expression levels of raft-resident proteins was also examined using spectral count quantitation. A list of five proteins has been identified for further validation by targeted analysis. We conclude that ATP Binding Cassette Transporter A1 regulates the lipid raft proteome of the resting and LPS-stimulated macrophage.

NIEHS
**Freudenberg, Johannes**
Research Fellow
Informatics/Computational Biology
*A meta-analysis reveals novel regulators required for mouse embryonic stem cell self-renewal*
Embryonic stem cells (ESCs) can self-renew indefinitely and can differentiate into all derivates (cell types) of the three primary germ layers. Thus, they could one day be exploited for transplantation therapies where a population of cells compromised by a disease/injury could be replaced with new functional cells. Successful development of such therapies would require a complete understanding of the transcriptional program that maintains the pluripotent genome in a stable state of self-renewal, while allowing rapid induction of alternate transcriptional programs to initiate differentiation. Master transcription factors Oct4, Sox2, cMyc, and Klf4 are necessary for the induction and maintenance of pluripotency. RNAi screens of about 16K genes in mouse ESCs (from 3 studies) collectively have revealed ~400 additional genes with roles in ESC maintenance. While these genome-wide screens were similarly designed and targeted similar sets of genes, there is almost no agreement between the sets of genes reported as required for ESC maintenance. The presence of unique hits in each study suggests that none of these screens have reached saturation and that many additional genes with roles in ESC maintenance remain to be discovered. In an effort to identify the set of all genes required for ESC maintenance, we developed a robust meta-analytic approach to integrate 68 previously published microarray gene expression datasets in mouse ESCs and differentiated cells to generate a list of genes, ranked by a metric quantifying the likelihood of their requirement to maintain ESCs. Top-ranked genes were significantly enriched for known ESC maintenance genes suggesting that genes ranked higher are good candidates for identifying novel ESC regulators. We used RNAi screens to knock-down (KD) 25 previously unscreened genes ranked in the top 500, and discovered 6 novel regulators required for ESC maintenance including Tet1, an enzyme mediating the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), one uncharacterized transcription factor, two genes involved in rRNA synthesis, one gene known to interact with histones H1 and H3, and one gene involved in histone deacytylation. Our gene expression profiling revealed that at least 2 out of the 6 genes are part of transcriptional circuit controlled by Oct4-Sox2-Nanog. Currently, we are performing high-throughput

sequencing to map 5mC and 5hmC levels to understand Tet1's role in regulating DNA demethylation in ESCs.

NIEHS
**GAO, HUIMING**
Research Fellow
Neuroimmunology
*HMGB1 (high-mobility group box 1) acts on microglia Mac1 (macrophage antigen complex 1) to mediate chronic neuroinflammation that drives progressive neurodegeneration*
What drives the gradual degeneration of dopamine neurons in Parkinson's disease (PD), the second most common neurodegenerative disease, remains elusive. Here, we demonstrated, for the first time, that persistent neuroinflammation was indispensible for such neurodegenerative process. 1-methyl-4-phenylpyridinium, lipopolysaccharide (LPS), and rotenone, three toxins often used to create PD models, produced acute but nonprogressive neurotoxicity in neuron-enriched cultures. In the presence of microglia (brain immune cells), these toxins induced progressive dopaminergic neurodegeneration. More importantly, such neurodegeneration was prevented by removing activated microglia. Collectively, chronic neuroinflammation may be a driving force of progressive dopaminergic neurodegeneration. On the other hand, ongoing neurodegeneration sustained microglial activation. Microglial activation persisted only in the presence of neuronal damage in LPS-treated neuron-glia cultures, but not in LPS-treated mixed-glia cultures. Thus, activated microglia and damaged neurons formed a vicious cycle mediating chronic, progressive neurodegeneration. Mechanistic studies indicated that HMGB1 (high-mobility group box 1), released from inflamed microglia and/or degenerating neurons, bound to microglial Mac1 (macrophage antigen complex 1) and activated NF-?B pathway and NADPH oxidase to stimulate production of multiple inflammatory and neurotoxic factors. The treatment of microglia with HMGB1 led to membrane translocation of p47 (a cytosolic subunit of NADPH oxidase) and consequent superoxide release, which required the presence of Mac1. Neutralization of HMGB1 and genetic ablation of Mac1 and gp91phox (the catalytic submit of NADPH oxidase) blocked the progressive neurodegeneration. Our findings indicated that HMGB1-Mac1-NADPH oxidase signaling axis bridged chronic neuroinflammation and progressive dopaminergic neurodegeneration, thus identifying a mechanistic basis for chronic PD progression.

NIEHS
**Hesse, Jill**
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
*Identification and Discovery of DNA Damage-Induced microRNA Expression Changes by Microarray and Next-Generation Sequencing in Wild-Type and ATM-Deficient Human Mammary Epithelial Cells*
MicroRNAs (miRNA) have been identified as important regulators of post-transcriptional gene expression and are critical in development, cell signaling pathways, and cancer progression. While many functions of miRNAs have been identified, it is far from clear the totality of roles these miRNAs are playing in cellular functions. The Ataxia-Telangictasia Mutated (ATM) gene product is a critical PI3-kinase related protein that has been shown to regulate DNA damage and cell cycle checkpoint responses. Individuals with deficiencies in ATM have severe ataxia and immunological problems and are predisposed to developing lympho-proliferative and breast cancers. Here we investigate the miRNA expression profile in response to both DNA damage induced by ionizing radiation (IR) and loss of ATM functionality. We utilize isogenic ATM wild type and ATM-deficient human mammary epithelial cell cultures (HME-CCs) in order to investigate the normal and ATM-dependent mammary epithelial response to DNA damage by investigating mRNA expression responses as well as to investigate miRNA

expression, with the ultimate goal of identifying profile signatures to identify IR-sensitive cells. We utilized two different methods to evaluate miRNA expression in HME-CCs, microarray and Next Generation sequencing. We are investigating both the best mechanism for evaluation of miRNAs as well as investigating the targets of statistically significant miRNAs by comparing the changes in expression of the miRNAs with changes in global mRNA gene expression in the same cells, in order to increase our understanding of the role of miRNA in DNA damage responses. We believe that direct comparison of the microarray and Next Generation sequence data will allow the most complete picture of miRNA regulation in response to DNA damage. Preliminary data analysis reveals both ATM-dependent and ATM-independent miRNA expression changes in response to DNA damage as well as ATM-dependent regulation of miRNA expression in the untreated conditions. Additionally, by exploiting the Next Generation sequencing technologies, we have identified possible novel DNA damage responsive small and miRNAs.

NIEHS
**Lai, Anne**
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
*DNA methylation primes the memory B cell epigenome for plasma cell differentiation*
Memory is a hallmark of adaptive immunity, wherein lymphocytes mount a superior response to a previously encountered antigen. Upon activation by antigen, naïve B lymphocytes undergo multiple rounds of cell division in the germinal center (GC) before differentiating into plasma cell effectors to secret high affinity antibodies. Alternative to a plasma cell fate, activated B cells also differentiate into memory B cells that can more rapidly acquire effector functions upon subsequent exposure to the same antigen. Memory B cells are quiescent and share a highly similar gene expression profile with their naïve counterparts. It remains incompletely understood how memory B cells can more rapidly turn on a plasma cell differentiation program. It has been speculated that epigenetic alterations in memory cells contribute to their functional distinction from their naïve counterparts. However, the nature and extent of epigenetic alterations in memory compartments remain poorly characterized. In this study, we characterized the DNA methylome of B cell subsets representing various stages of the immune response before and after antigen exposure in vivo. Naïve B cells prior to antigen exposure, activated B cells in GC, post GC-derived memory B cells and plasma cells were purified from inflamed human tonsils for isolation of genomic DNA. Methylated DNA fragments were captured using a methyl-CpG binding protein and analyzed using microarrays covering about 10 percent of the human genome. Comparison of the DNA methylome of B cell subsets indicated that immune activation led to widespread alterations of this epigenetic mark at both promoter-proximal and intergenic regions. These activation-induced DNA methylation changes generated a similar epigenetic signature in downstream memory B cells and plasma cells that is vastly different from naïve B cells. Differential methylated regions between naïve and memory B cells were enriched near genes with immune functions and contain B-cell specific transcription factor binding sites. We propose that the DNA methylation signature of memory B cells facilitates the recruitment of transcription factors upon antigenic re-challenge, thus allowing them to more rapidly turn on a plasma cell transcriptional program during a secondary immune response.

NIEHS
**Li, Hong**
Research Fellow
Immunology - Autoimmune
*Cyclooxygenase-2 (COX-2) Negatively Regulates IL-9+/CD4+ T cells (Th9) Differentiation during Allergic Lung Inflammation through down-regulation of IL-17RB*

Helper CD4+ T cell subsets, which include Th1, Th2 and Th17 cells, are key components of the adaptive immune response in rodents and humans. Recent data suggests that another distinct helper CD4+ T cell population, Th9 cells, which produce IL-9 and IL-10, exist under certain conditions and that Th2 cells can differentiate into Th9 cells. Our previous studies indicate that COX enzymes play an important role in regulating the Th1-Th2 balance in allergic lung diseases via inhibition of Th2 immune responses. However, the role of COX enzymes in Th9 differentiation is unknown. Herein, using COX-1-/-, COX-2-/- and wild type (WT) mice we investigated whether COX-1 and/or COX-2 were involved in regulating differentiation of Th9 cells during ovalbumin (OVA)-induced allergic lung inflammation. Following OVA sensitization/exposure, the percent of Th9 cells was significantly higher in lung (3.2±0.8 vs. 1.7±0.6), bronchoalveolar lavage fluid (BALF) (3.9±0.5 vs. 2.2±0.7), lymph nodes (27.2±5.1 vs. 14.3±5.1) and blood (20.9±1.9 vs. 13.6±2.3) of COX-2-/- mice, but not COX-1-/- mice, relative to WT controls (all p<0.05). In addition, BALF and serum IL-9 levels were dramatically increased in COX-2-/- mice compared to WT controls. In vitro, Th9 cell differentiation following treatment of naïve CD4+ T cells with TGF-beta and IL-4 was significantly higher in COX-2-/- cells compared to WT cells. Prostaglandin (PG) D2 and PGE2 significantly down-regulated PU.1 expression and inhibited Th9 cell differentiation from naïve CD4+ T cells. PGD2 and PGE2 also inhibited IL-17RB expression, but not IL-17RA, suggesting that PGs directly regulate Th9 cell differentiation through down-regulation of IL-17RB. PGD2 and PGE2 signal through specific EP and DP cell surface receptors. Importantly, Th9 cell differentiation was increased in CD4+ T cells from EP2-/- mice or after siRNA knockdown of DP2 receptor. More importantly, we found human asthmatic patients have significantly increased IL-9+/CD4+ T cells compared with normal controls. PGD2 dramatically inhibited Th9 cell differentiation from human naïve CD4+ T cells. Together, these results indicate that COX-2 inhibits Th9 cell differentiation from naïve CD4+ T cells via PG signaling and regulation of IL-17RB expression in CD4+ T cells. Therefore, targeting COX-2 and/or prostaglandin signaling pathways may represent a novel approach to the treatment of human allergic lung disease.

NIEHS
**Lichti-Kaiser, Kristin**
Postdoctoral Fellow
Endocrinology

*The Role of Glis3 in the Development of Functional Pancreatic Beta-cells and Diabetes*

Gli-similar (GLIS) 3 belongs to a subfamily of Krüppel-like zinc transcription factors that are related to members of the Gli and Zic family. In recent years, several genes including GLIS3 have been associated with risk for type-1 and type-2 diabetes and genetic aberrations in the GLIS3 gene have been associated with a syndrome characterized by neonatal diabetes and congenital hypothyroidism. We have shown that Glis3 KO mice die at post-natal day 3 from neonatal diabetes as evidenced by hyperglycemia and hypoinsulinemia. This phenotype is due to a dramatic loss of insulin-secreting beta-cells in contrast to a smaller relative loss of other endocrine cell types. In addition, Glis3 regulates insulin gene expression in mature beta-cells, indicating that Glis3 plays a key role in both the development and function of mature pancreatic beta-cells. However, the spatial and temporal expression of Glis3 during pancreas development and the mechanism by which Glis3 contributes to the development and maintenance of functional beta-cells are unknown. We have generated two pancreas-specific Glis3 KO mouse models. Glis3 fx/fx mice were crossed with mice expressing cre-recombinase under the control of the Pdx or Insulin gene promoters, expressed at early and late time-points during pancreas development, in order to generate pancreas- and beta-cell-specific KO models respectively. Pdx(Glis3) KO mice develop a delayed-onset diabetic phenotype at 2-3 months of age as evidenced by hyperglycemia, hypoinsulinemia, and loss of white adipose tissue whereas Ins(Glis3) KO mice do not develop overt diabetes. Gene expression profiling and immunofluorescence analysis demonstrated that the expression of insulin was significantly decreased at 2 months, but not at 2 weeks, of age in Pdx(Glis3) KO mice. The

expression of other pancreatic hormones and transcription factors important for endocrine cell development, including Ngn3 and Pdx1, was not significantly different than that of WT littermates, indicating that, unlike in the whole-body KO, there is not a significant loss of beta-cells in the Pdx(Glis3) KO mice. The Pdx(Glis3) KO mice appear to provide an excellent model to examine various therapeutic strategies for diabetes. Our study shows that Glis3 has multiple critical functions in the pancreas and suggests that Glis3 may provide a new therapeutic target to intervene in diabetes.

NIEHS
**Narlikar, Leelavati**
Other
Informatics/Computational Biology
*Genome-wide characterization of CTCF's role as an enhancer-blocker*
Insulators are DNA elements which when bound by proteins regulate gene expression by blocking interactions between enhancers and promoters, thus limiting the activity of enhancers to certain functional domains. CTCF, the only known insulator-binding protein in vertebrates, has been shown to play diverse regulatory roles including enhancer blocking and genomic imprinting. The region containing H19 and Igf2 genes separated by imprint control region (ICR/insulator) is a well studied locus, where CTCF binds the ICR in a DNA methylation-sensitive manner. Normally, CTCF binds the unmethylated maternal allele ICR, blocking the enhancer downstream of H19 from interacting with Igf2 promoter, thus silencing Igf2 but not H19. On the paternal allele, CTCF is inhibited from binding the methylated ICR, permitting the enhancer to interact with Igf2 promoter, resulting in paternal specific expression of Igf2. Hyper- or hypo-methylation of ICR inhibits/permits CTCF binding resp., resulting in aberrant Igf2 expression associated Beckwith-Wiedemann and Silver-Russell syndromes. In this study, we sought to investigate CTCF's enhancer-blocker role on a genome-wide scale. Previously we had used ChIP-Seq to map CTCF binding sites in HeLa cells, a cancer cell-line. Here, we used ChIP-Seq to map enhancer elements (marked by co-activators and enhancer-specific histone marks) in HeLa cells. We developed an elegant generative grammar-based computational approach to systematically categorize adjacent gene-pairs into 4 groups: (A) those separated by a CTCF site and an enhancer, (B) those separated only by a CTCF site, (C) those separated only by an enhancer, and (D) those that have neither in between. We hypothesized that adjacent genes in group A will be differentially expressed: the enhancer will activate only the gene immediately next to it, with the CTCF-DNA interaction blocking its effect on the other gene. We expected no such effect for gene-pairs in groups B, C, or D. Microarray expression data in HeLa cells revealed that this is indeed the case. To ensure that the observed differences are due to CTCF's direct role is enhancer-blocking, we are currently employing RNAi to knock-down CTCF in an effort to show that the loss of CTCF binding abolishes the expression divergence observed for gene-pairs in group A by essentially converting them into gene-pairs belonging to group C. Our findings would provide a compelling case for CTCF's role as an enhancer-blocker on a global scale.

NIEHS
**SAITO, KOSUKE**
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
*The role of the potassium channel KCNK1 in the sexual dimorphic centrilobular hypertrophy induced by phenobarbital in mouse liver*
The liver is one of the organs that exhibit sexually dimorphic responses against therapeutics and xenobiotics. For example, the susceptibility of non-genotoxic liver tumor development caused by phenobarbital (PB) is higher in male than female mice. Although PB activates the constitutive active/androstane receptor (CAR) to cause the male-predominant tumor development, the expression

of CAR and the PB-caused induction of a classical CAR target gene Cyp2b10 are sex-independent in mouse liver. Therefore, the molecular mechanism of the male-predominant tumor development is not well understood. Given the fact that hypertrophy and hyperplasia precede tumor development, we have now identified KCNK1 (an inwardly rectifying potassium ion channel) as a CAR target that may be responsible for the male-predominant development of liver tumors. First, the expression of KCNK1 was induced by PB treatment only in male but not in female liver. Second, the induction of KCNK1 was no longer observed in livers of Car-/- male mice. Promoter analysis demonstrated that CAR activated the Kcnk1 promoter through a 97-bp DNA sequence (97-bp RE). ChIP assay revealed that PB treatment enhanced the binding of CAR on the 97-bp RE only in male but not female liver. These results imply that the male-specific binding of CAR on the 97-bp RE is responsible for the male-specific activation of the Kcnk1 gene by PB treatment. In general, potassium channels are reported to play a role in regulating carcinogenesis, apoptosis, and cellular volume. Therefore, we used histological analyses to examine whether KCNK1 plays a role in the PB-induced development of liver tumors. KCNK1 was induced in the hepatocytes around the central veins but not in those around the portal veins of PB-treated male liver, while no significant induction of KCNK1 was observed in female liver. This male-specific, centrilobular hepatocyte-specific induction of KCNK1 was consistent with the incidence of the PB-induced centrilobular hypertrophy. Moreover, Kcnk1-/- male mice exhibited a significant decrease in the PB-induced centrilobular hypertrophy. Although the KCNK1-regulated hypertrophy is not directly linked to tumor development, our present experimental results suggest that KCNK1 can be a possible CAR target for sexually dimorphic non-genotoxic carcinogenesis induced by PB. In addition, our results provide an animal model for further investigations to delineate the molecular mechanism of this non-genotoxic tumor development.

NIEHS
**Takeda, Yukimasa**
Visiting Fellow
Cell Biology - General
*Retinoic acid-related orphan receptor gamma, RORgamma, coordinates the circadian regulation of energy homeostasis through the control of hepatic lipid and glucose metabolism*
Defects in circadian rhythm have increasingly become a risk factor for several pathologies, including metabolic syndrome and cancer. We reported that RORgamma exhibits a strong oscillatory pattern of expression during circadian time and modulates the expression of several circadian clock genes in peripheral tissues. In this study, we reveal that RORgamma is a key transcription factor in linking the regulation of circadian rhythm to the control of lipid and glucose homeostasis. We demonstrated that energy expenditure of RORgamma-deficient (RORgamma-KO) mice as measured by oxygen consumption is strongly decreased at night time, a period at which RORgamma is most highly expressed, and not during day time. Lower respiratory exchange rate (RER) in RORgamma-KO mice suggests a preference for lipid consumption over glucose consumption in order to produce energy. Interestingly, in RORgamma-KO mice fed with a high-fat diet (HFD) the decreased energy expenditure is restored, which is likely due to the increased consumption of dietary fatty acids in RORgamma-KO mice compared to WT mice. The serum triglyceride concentration in the RORgamma-KO mice was lower than WT mice, while serum ketone bodies, largely generated by the liver, are increased in a circadian time-dependent manner. We next showed that RORgamma directly and indirectly regulates hepatic gene expression critical in lipid and glucose metabolism. In particular, RORgamma predominantly regulates circadian expression of Elovl3, elongation of very long chain fatty acids-like 3, and Avpr1a, arginine vasopressin receptor 1a, each of which regulates fatty acid biosynthesis and lipolytic activity. Also, the expression of Srebp1c and G6Pase is reduced in RORgamma-KO liver in vivo, but not in RORalpha-deficient liver, implicating that efficiency of lipogenesis and glyconeogenesis may be reduced. ChIP analysis using liver

tissues showed that Elovl3 and G6pase genes are directly regulated by RORgamma through ROR-responsive elements (ROREs). In addition, microarray result shows increased gene expression of other several enzymes critical for peroxisomal and mitochondrial beta-oxidation as well as ketogenesis in RORgamma-KO liver, which supports the observation that RORgamma-KO mice more consume lipid for energy production. Our study identifies a novel physiological function of RORgamma in the coordination of the circadian regulation of energy homeostasis through hepatic lipid and glucose metabolism.

NIEHS
**Tumbale, Percy**
Postdoctoral Fellow
Protein Structure/Structural Biology
*Structural Basis of DNA Ligase Proofreading by Aprataxin with insights into AOA1 Neurodegenerative Disease*
Maintenance of genomic integrity is completed in the ultimate step of DNA replication and repair transactions when eukaryotic ATP-dependant DNA ligases seal DNA nicks in a three step reaction: 1) adenylation of a ligase active site lysine, 2) transadenylation of DNA 5'-phosphate, and 3) phosphodiester bond formation with AMP release. This biologically critical process can fail at the last step, leaving 5'-adenylated DNA termini when ligases engage nicks harboring DNA-distorting adducts and DNA repair metabolites including common products of abundant cellular oxidative DNA damage. DNA 5'-adenylation must be reversed to prevent persistence of DNA single strand breaks (SSBs) and genome instability. To protect genomic integrity, Aprataxin (APTX) repairs damaged 5'-adenylated DNA termini arising from failed DNA ligation events. The importance of APTX DNA processing functions in mammals is underscored by the fact that mutations in human Aprataxin (APTX) are linked to the autosomal recessive neurological disorder Ataxia with Oculomoter Apraxia 1. However, due to a lack of detailed structural information, the molecular basis for APTX catalytic reversal of 5' adenylation damage, and how APTX is inactivated in the neurodegenerative disorders remain largely unknown. Here, we report X-ray crystal structure of a Schizosaccaromyces pombe APTX/dsDNA/Amp ternary complex that show how structural fusion of a HIT (histidine triad) nucleotide hydrolase fold with a Zif-268 related Zn-finger DNA binding scaffold assembles the APTX active site and dsDNA interaction surfaces. APTX-DNA complex structure illustrates how APTX senses DNA ends or nicks by capping the exposed base stack with a helical wedge. Employment of paired aromatic paddles and a conserved APTX "FPK-pivot" motif facilitates rotation of DNA 5'-adenylate orthogonal to the dsDNA duplex axis and into the active site pocket for de-adenylation repair. To dissect molecular determinants of APTX substrate recognition and catalysis we generated an extensive set of single amino acid substitutions based on APTX structures. Our results illustrate that obstruction of APTX DNA binding surface specifically impairs catalysis on adenylated DNA substrates, but not on nucleotide substrates such as Ap4A. Structures and mutagenesis support a HIT-Znf catalytic mechanism for AMP-adduct removal, and reveal mutations impacting protein folding, the active site pocket, and FPK-pivot underlie APTX dysfunction in neurodegenerative disease.

NIEHS
**Verhein, Kirsten**
Postdoctoral Fellow
Genomics
*Candidate susceptibility genes in a murine model of RSV-induced bronchiolitis*
Respiratory syncytial virus (RSV) is the most common cause of serious lower respiratory illnesses in infants and small children; almost all children have been infected with RSV by age 2. Globally, an estimated 1 million children die annually from severe RSV disease. While genetic association studies have been reported for candidate susceptibility genes for RSV disease, genome wide association studies

(GWAS) have not been performed and no biomarkers exist for predicting RSV disease severity. We use a combined genetics and genomics approach to understand the genetic basis of RSV disease susceptibility. We screened 32 inbred mouse strains for response to RSV infection by measuring disease phenotypes, including inflammatory cells and protein concentration in bronchoalveolar lavage, airway mucus, and RSV viral load. Differential disease phenotypes were analyzed using SNPster (Novartis), a GWAS algorithm that identifies statistically significant associations between haplotype and phenotype. Statistically significant and suggestive quantitative trait loci (QTLs) were found for all disease phenotypes. A candidate gene Marco (macrophage receptor with collagenous structure) was identified on chromosome 1, and validated using Marco-/- mice. Furthermore, a non-synonymous coding single nucleotide polymorphism (SNP) was significantly associated with severe disease in a case-control cohort of infants with mild and severe RSV-induced lung disease. Microarray mRNA expression data after RSV infection from lungs of resistant and susceptible mice identified gene transcripts expressed only in susceptible strains, including tumor necrosis factor alpha (Tnf). To identify gene transcripts that predict response to RSV, baseline lung gene transcript expression for 29 inbred mouse strains (Novartis) was correlated to phenotype data from the RSV strain screen using linear regression. These studies identified a battery of gene transcripts that were differentially expressed at baseline and significantly correlated with RSV disease severity including Il1rn (IL-1 receptor antagonist). A test set of gene transcripts developed in the mouse model of RSV disease is being tested in samples from the case-control RSV disease cohort to determine predictability of disease severity in humans. Together, these approaches have identified genetic markers of susceptibility to RSV disease to predict severe responders and potentially provide more effective therapeutic targets.

NIEHS
**Wang, Xueqian**
Research Fellow
Pharmacology and Toxicology/Environmental Health
*Nuclear Factor E2-Related Factor-2 (Nrf2) Regulates P-glycoprotein Expression at the Blood-Brain Barrier (BBB) by Acting Through p38 MAP Kinase*
At the BBB, the ATP-driven efflux pump, P-glycoprotein (Pgp) is a major impediment to CNS pharmacotherapy. Signals that modulate Pgp transport function are complex and not fully mapped. Recent studies show that Pgp is upregulated by xenobiotics acting through nuclear receptors, e.g., aryl hydrocarbon receptor (AhR). Here we show that ligands for Nrf2 increase Pgp-mediated transport and transporter protein expression in rat brain capillaries. Nrf2 senses oxidative/electrophilic stress and induces multiple cytoprotective proteins, including antioxidant and glutathione generating enzymes, but its ability to modulate expression of transport proteins is largely unexplored. Under basal conditions, Nrf2 is retained in the cytosol. Upon activation, Nrf2 translocates to the nucleus, where it forms a heterodimer with a small Maf protein, binds to an antioxidant response element (ARE) and induces transcription. We found that the promoter region of rat Pgp contains multiple Nrf2 binding sites. We used freshly isolated rat brain capillaries, a fluorescent Pgp substrate and confocal microscopy to monitor chages in Pgp transport activity. Exposing capillaries to the Nrf2 ligand, sulforaphane (SFN, 0.1-10 μM), a naturally occurring compound present in cruciferous vegetables, increased Pgp activity in a concentration-dependent manner. Increased transport was observed with 2 h SFN exposure and reached a maximum with 3h of exposure. Inhibiting transcription with actinomycin D or inhibiting translation with cycloheximide abolished SFN-induced upregulation of transport. Another Nrf2 activator, tert-butylhydroquinone (tBHQ), widely used in food preservatives, also significantly increased Pgp transport activity. Exposing rat brain capillaries to SFN (1-10 μM) caused a concentration-dependent increase of Pgp protein expression assayed by Western blot. Electrophoretic Mobility Shift Assay (EMSA) detected binding of Nrf2 to ARE in nuclei from rat brain capillaries exposed to SFN. Pretreament with

SB203580, a p38 mitogen-activated protein kinase inhibitor, abolished SFN- and tBHQ- induced upregulation of Pgp transport, while inhibitors of MEK and PI3 kinase were without effect. These results implicate p38 signaling in Nrf2 induction of Pgp activity. Thus, the BBB is tightened selectively by dietary constituents that are Nrf2 ligands, providing increased neuroprotection but at the expense of reduced penetration of certain therapeutic drugs.

NIEHS
**Wang, Huanchen**
Postdoctoral Fellow
Protein Structure/Structural Biology
*Substrate Specificity and Catalysis Mechanism of Inositol Pyrophosphate Kinase*
The inositol pyrophosphates, IP7 and IP8, consist of phosphates and diphosphates closely packed around a six-carbon inositol ring. These molecules regulate a variety of cellular processes including apoptosis, vesicle trafficking, cytoskeletal dynamics, exocytosis, telomere maintenance, and adaptations to environmental stress. In this study we have asked how the reaction mechanism and stringent substrate specificity of the IP7 kinase (PPIP5K) has evolved despite the steric bulk and intense electronegativity of multiple phosphates in such a confined space. The answers to these questions come from our providing the first description of the crystal structure of an enzyme that synthesizes an inositol pyrophosphate. We have obtained 14 separate structures of PPIP5K to 1.7-2.0 A resolution in binary, ternary or quaternary complexes with either nucleotide, substrate, transition analog or product. Specificity of docking of either IP6 or 5-IP7 into the substrate binding pocket is ensured by an array of positively charged lysine and arginine residues. The importance of these key residues to substrate binding was verified by mutagenesis and by kinetic studies. ATP was deeply buried with only 9% solvent-accessible indicate that it is difficult to exchange with the bulk phase. The various structure complexes with the transition state analog, the reactant, and the product, provide possible snapshots of dynamic reaction steps. After the IP7 substrate is bound, slight conformational changes compress the distance between the IP7 and ATP by about 1 A, and this facilitates nucleophilic attack to the gamma phosphate of ATP, and then the IP8 product leaves the active site. The magnesium atom in the transition state analog is 2.3A from the donor and 1.9A from the acceptor oxygen atoms and the angle is 166 degree among the three atoms, indicating a partial associative character of the transition state. This direct-in-line phosphoryl transfer mechanism rules out the possibility of a phosphoenzyme intermediate participating in the reaction. Lys-248 emerges as a key catalytically-essential residue and we find that it is not just spatially conserved across inositol kinases but also in cAMP dependent protein kinase A. Our studies not only provide us with an understanding on the catalytic mechanism but also they reveal a novel evolutionary link between inositol kinases and protein kinases in term of the phosphoryl transfer mechanism.

NIEHS
**Winuthayanon, Wipawee**
Research Fellow
Endocrinology
*Role of epithelial estrogen receptor alpha in the oviduct during fertilization and embryo development*
Mammalian fertilization and preimplantation embryo development occur in the oviduct within a microenvironment strictly controlled by steroid hormones. Changes in oviductal cytology in each cell compartment are the most apparent during the estrous stage of the ovarian cycle, immediately prior to fertilization. The goal of this study was to elucidate the role of estrogen receptor alpha (ERa) in oviduct epithelium during fertilization and preimplantation embryo development. We previously generated a conditional knockout (cKO) mouse lacking ERa in female reproductive tract epithelium by crossing

Wnt7a-Cre with Esr1-floxed mice. The cKO females were completely infertile in part because of inability to achieve uterine receptivity. To determine if oviductal dysfunction also contributed to their infertility, we first documented by immunohistochemistry that ERa was not expressed in oviductal epithelium of the cKO mice. We then examined ovulated oocytes and evaluated preimplantation embryo development in vivo by flushing embryos from the oviduct and uterus on days 1, 2 and 4 of pregnancy. The cKO mice ovulated similar numbers of morphologically normal appearing eggs as control littermates, and one-cell stage embryos were flushed from the oviduct on pregnancy day 1. However, there were no 2-cell embryos or blastocysts on day 2 or day 4 of pregnancy, respectively, although a few fragmented embryos and empty zona pellucidae were observed. When the one-cell embryos were cultured in vitro, very few embryos collected from cKO oviducts progressed to the expanded blastocyst stage whereas most embryos collected from control oviducts developed into hatching blastocysts. These results indicate that lack of ERa in the oviductal epithelium results in alterations in the oviductal microenvironment that completely disrupt preimplantation embryo development beginning at the one-cell stage. This is a highly novel and unexpected regulatory phenomenon of epithelial ERa in the female reproductive tract that will provide information important for understanding mechanistic actions of estrogenic environmental chemicals and their impact on very early mammalian pregnancy as well as the roles of ERa in human fertility.

NIEHS
**Xu, Mengyuan**
Research Fellow
Informatics/Computational Biology
*coMOTIF: A Mixture Framework for Identifying Transcription Factor and a Co-regulator Motifs in ChIP-seq Data*
ChIP-seq data should be enriched in binding sites for the transcription factor that was immunoprecipitated. Some of the sequences may also contain binding sites for a transcriptional co-regulator. Most existing motif discovery algorithms such as MEME identify one motif at a time. Methods that consider the joint distribution of multiple motifs in the sequences are typically designed to identify clusters of binding sites (a cis-module) that are close to each other, e.g., in a 100- or 200-base pair window. These methods are well-suited for promoter sequences of co-expressed genes on which multiple transcription factor binding sites tend to co-localize. However, they may not be ideal for sequences without well-structured cis-modules such as ChIP-seq data. We developed a finite mixture framework to simultaneously estimate the position weight matrices (PWMs) of both primary and co-regulator motifs and determine which sequences contain both motifs, either single motif, or neither of them. The co-existence of the two motifs in a sequence is modeled by their joint bivariate distribution whereas each single motif is modeled univariately. We use the EM algorithm to iteratively update the parameters of each motif's position weight matrix and the mixing proportions. We compute the posterior probabilities that any given sequence contains both motifs, either motif, or neither, allowing both forward and reverse-compliment orientation. We tested our method on simulated ChIP-seq datasets and showed that it performed better than repeated application of MEME in predicting which sequences contained motifs, and better than a cis-module-based method in estimating the PWMs. We applied our method to a mouse liver Foxa2 ChIP-seq dataset involving ~12,000 400-bp sequences. We identified co-occurrence of Foxa2 with Hnf4a, Cebpa, E-box, Ap1/Maf or Sp1 motifs in ~5-33% of these sequences. All these secondary motifs are either known liver-specific transcription factors or factors known to play an important role in liver function. This framework is novel in considering the joint distribution of the two motifs within a sequence and estimating the proportion of sequences containing either one or both motifs. Our method is available as a software tool, coMOTIF, and is applicable to large-scale genomic ChIP-seq data for the discovery of a transcription factor and a co-regulator motif.

NIEHS
**Yin, Zhengyu**
Visiting Fellow
Carcinogenesis
*RAP80 Plays a Critical Role in Maintaining Genomic Stability and Tumor Suppressing*
The DNA damage response (DDR) coordinates activation of cell cycle checkpoints, apoptosis and DNA repair networks, to ensure accurate repair and genomic integrity. Phosphorylation of the histone H2A variant, referred as gamma-H2AX, is one of the initial signaling events which sense DNA double strand breaks and is required for the subsequent recruitment of many DDR proteins to sites of DNA damage. Upon DNA damage by ionizing irradiation (IR), the ubiquitin interaction motif (UIM)-containing protein RAP80 binds to poly-ubiquitin chain of H2A and gamma-H2AX, and mediates DNA repair events by recruiting DDR mediators and effectors, especially BRCA1. Knockdown of RAP80 expression in vitro greatly impairs the localization of BRCA1 to the IR-induced foci and therefore increases susceptibility to IR or other DNA damaging agents. Based on these observations, we hypothesized that RAP80 functions as a tumor suppressor gene and that deficiency in RAP80 may lead to genomic instability in vivo and promote cancer. In this study, RAP80 knockout (KO) mice were generated and characterized. In contrast to the embryonic lethal phenotype in BRCA1 KO mice, RAP80 KO mice are viable and do not exhibit any major anatomical defect. However, we showed that mouse embryonic fibroblasts (MEFs) from RAP80 KO mice exhibited slower rate of proliferation as well as a higher percentage of premature senescence compared to wild type (WT) MEFs. RAP80 KO MEFs also showed increased spontaneous and IR-induced genomic instability, which led to prolonged G2/M cell cycle arrest. Moreover, RAP80 KO MEFs contained a higher percentage of spontaneous gamma-H2AX positive cells compared to WT MEFs and IR induced more nuclear fragmentation in RAP80 deficient MEFs than WT controls. Loss of RAP80 increased sensitivity to IR in vivo and in vitro while IR-induced gamma-H2AX foci disappeared at a slower rate in RAP80 KO MEFs. In addition, in response to IR, p53 and several p53 targeting pro-apoptotic genes were activated in absence of RAP80. Tumorigenesis studies suggested that RAP80 KO mice exhibited an increased susceptibility to the spontaneous development of lymphoma and the development of DMBA-induced mammary gland cancer. Altogether, these data indicate that RAP80 functions as a tumor suppressor gene and that deficiency of RAP80 leads to genomic instability and predisposition to cancer.